INHIBITION OF INFLUENZA VIRUS AND HUMAN PARAINFLUENZA I VIRUS INFECTION BY THE PROTEASE INHIBITOR HAI-2

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Soreen Yuenting Cyphers

August 2014

ABSTRACT

Influenza virus remains a significant concern to public health, as there is a continued potential for a high fatality pandemic. Multiple strains of influenza have emerged that are resistant to antiviral therapeutics currently on the market. One approach to an antiviral therapeutic is to inhibit cleavage activation of the influenza virus hemagglutinin (HA). The viral HA must be cleaved by host cell proteases in order to fuse with the cell membrane during virus entry and is most likely driven by extracellular or membrane bound trypsin-like proteases. One such inhibitor of these proteases is hepatocyte growth factor activator inhibitor-2 (HAI-2). We show that HAI-2 has the potential to be used as an antiviral therapeutic for influenza and other viruses with similar entry mechanisms

BIOGRAPHICAL SKETCH

Soreen Cyphers earned her Bachelor of Science degree in Biochemistry from Syracuse University in 2012. In 2012, she joined the Biochemistry, Molecular, and Cell Biology program at Cornell University and will be leaving with a Master of Science degree in Biochemistry.

Soreen Cyphers has been the recipient of the iLearn grant and Birge Award for Exceptional Performance in Biochemistry Research at Syracuse University. At Cornell, she was awarded the NIH Cellular and Molecular Biology Training Grant. While pursuing her degree, she worked as a teaching assistant for the department of Undergraduate Biology.

DEDICATION

I would like to dedicate my thesis to my parents, Scott and Michelle Cyphers, for supporting me through all my decisions and to my boyfriend, Dong Lee, for always being there.

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Gary Whittaker, my research advisor. I want to thank him for allowing me to be a part of this lab, and for all of the support he has given me. Dr. Whittaker has been a constant source of research advice and was always ready to help me when needed. He has helped me become a better researcher, and it has been a privilege to work with him. I would also like to thank Dr. William Brown for being my committee member and supporting me in my research.

Thank you to all of the members of the Whittaker lab. I appreciate all of the help you have given me during my time in the lab and all of the fun times we had together. Dr. Victor Tse, Dr. Jean Millet, Dr. Lisa Bolin, Dr. Michele Bialecki, Beth Licitra, Kelly Sams, and Wendy Wingate.

I would also like to thank the Biochemistry, Molecular, and Cell Biology program at Cornell University for giving me the opportunity and skills I needed to succeed, as well as the Department of Microbiology and Immunology.

Finally, I would like to thank my family and friends for all of the support you have given me. To my parents, Scott and Michelle, I appreciate everything you've done for me. Also, thank you to my boyfriend, Dong Lee, for your constant encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

WHO	World Health Organization
NP	Nucleoprotein
M	Matrix
HA	Hemagglutinin
NA	Neuraminidase
M1	Matrix 1
M2	Matrix 2
TTSP	Type II Transmembrane Serine Protease
HAT	Human Airway Trypsin-like Protease
RNP	Ribo-nucleoprotein
FDA	Food and Drug Administration
HPIV-1	Human Parainfluenza I Virus
HN	Hemagglutinin-neuraminidase
F	Fusion protein
N	Nucleoprotein
P	Phosphoprotein
L	Large protein
HAI-1	Hepatocyte growth factor activator 1
HAI-2	Hepatocyte growth factor activator 2
HGFA	Hepatocyte growth factor activator
LB	Luria broth
PBS	Phosphate buffered saline
VSV	Vesicular stomatitis virus
FFU	Foci fluorescent unit
FPLC	Fast protein liquid chromatography

Abstract

Influenza virus remains a significant concern to public health, as there is a continued potential for a high fatality pandemic. Multiple strains of influenza have emerged that are resistant to the antiviral therapeutics currently on the market. One approach to an antiviral therapeutic is to inhibit cleavage activation of the influenza virus hemagglutinin (HA). The viral HA is synthesized as a fusion-inactive precursor (HA0) that must be cleaved by host cell proteases in order to fuse with the cell membrane during virus entry. HA cleavage is most likely driven by extracellular or membrane bound, trypsin-like proteases. By targeting host cell proteases that cleave the HA protein, cell infection by influenza virus could be stopped.

An inhibitor of serine proteases is hepatocyte growth factor activator inhibitor-2 (HAI-2). HAI-2 contains a Kunitz-type inhibitor domain that has been found to be a potent inhibitor of a number of trypsin-like serine proteases. HAI-2 can be used to inhibit cleavage of HA *in vitro*. HAI-2 administration to mice shows decreased viral titer in mice. Finally, data showing HAI-2 prevention of spread of human parainfluenza virus-1, which has a similar activation mechanism to influenza, shows promise in utilizing HAI-2 as a broad-range antiviral therapeutic for viruses similar to influenza.

Background

Influenza Virus

Influenza causes a contagious respiratory illness that infects the upper respiratory tract and sometimes the lower respiratory tract. Common symptoms include sneezing, headache, coughing, and fever (1). Children, the elderly, and the immunocompromised are most at risk for more serious complications such as pneumonia and dehydration, which can result in hospitalization or death (1). The World Health Organization (WHO) estimates that 5% to 10% of adults and 20% to 30% of children are infected by influenza (1). In the United States alone, over 200,000 people are hospitalized due to influenza annually (1). As well as health concerns, influenza also exerts a significant economic burden on healthcare systems. In the United States, annual direct and indirect costs related to influenza due to hospitalization and productivity losses was estimated to be 16.3 billion dollars in 2003 (2).

There are three types of influenza virus: influenza A, influenza B, and influenza C (1). They are classified by antigen variation in their nucleoprotein (NP) and matrix (M) protein (1). Influenza A and B are responsible for most of the infections observed in humans, while influenza C is antigenically stable and only causes a mild illness (1). However, influenza A raises the most public health concern among the three.

Influenza A is found in a variety of organisms, from birds to pigs to humans (3). It is divided into subtypes based on two of its surface proteins, hemagglutinin (HA) and neuraminidase (NA). There are currently 18 known HA subtypes and 11 known NA subtypes (3). The large variance of influenza A subtypes occurs because it can undergo genetic changes via antigenic shift or antigenic drift. Antigenic drift occurs when mutations occur in the genome, changing the amino acids in the antigenic portions of the HA and NA

proteins (4). When two strains of influenza infect the same host, antigenic shift can occur. Antigenic shift occurs when two different strains of influenza exchange their genetic material. Since influenza has a segmented genome, it is able to recombine creating a new subtype (4). This reassortment introduces a novel virus that can occasionally become more virulent and can cause influenza pandemic (4). A pandemic occurs when a new influenza strain enters the population that is different from seasonal virus strains, meaning the population has not built up any immunity against it. In the last century, there have been four pandemics caused by influenza A: the Spanish Flu of 1918, the Asian Flu of 1957, the Hong Kong Flu of 1968, and the 2009 H1N1 "swine flu" (1).

Influenza Structure and Replication

Influenza is a segmented, negative sense, single-stranded RNA virus (1). Its segmented genome is enclosed within a host-derived lipid membrane, which has a variety of surface proteins embedded in it (Figure 1.1A). The two major surface proteins are HA and NA. HA is important in viral entry and fusion with the host cell membrane (5). It originally exists in a pre-cleaved form, HA0 (1). The NA protein is responsible for viral release from the cell (1). There are also a variety of other proteins that contribute to the structure and replication of the influenza virus. The NP contributes to the structure of the viral capsid. The matrix 1 (M1) protein forms a coat under the viral envelope that mediates the encapsulation of the RNA-nucleoprotein cores into the membrane envelope (1). The matrix 2 (M2) protein is an ion channel, which helps to maintain the pH across the viral membrane during entry and maturation of the viral capsid (1).

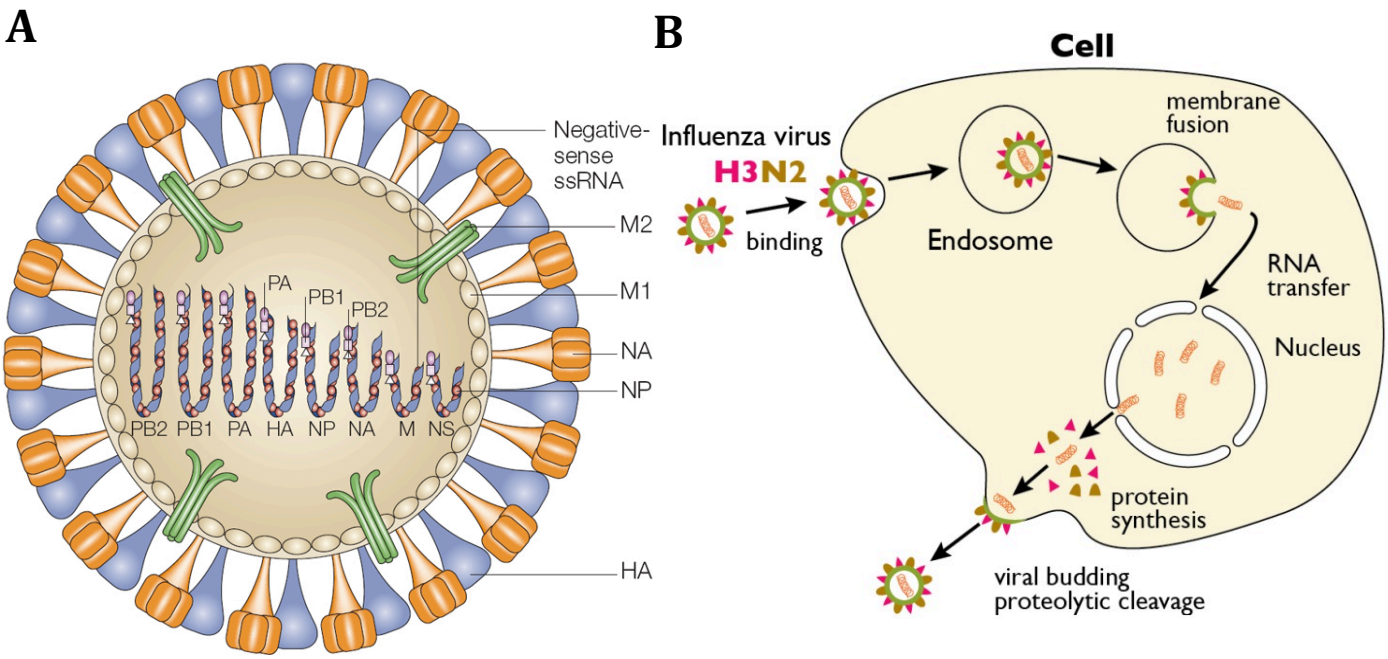


Figure 1.1 A) Structure of influenza virus depicting segmented viral genome and proteins. (modified from ref. 6) B) Influenza replication pathway: binding to cell membrane, fusion and release of viral genome, protein synthesis, budding and release from cell membrane (modified from ref. 7)

In order for an influenza virus particle to enter a host cell, it must first bind to sialic acid glycoproteins found at the surface of host cells (Figure 1.1B) (5). The HA protein binds to sialic acid, initiating receptor-mediated endocytosis and bringing the virus to the endosome (8). Before the virus particle is enveloped by the endosome, the HA protein is cleaved by host cell proteases (8).

A variety of type II transmembrane serine proteases (TTSP), such as TMPRSS2 and human airway, trypsin-like protease (HAT) have been shown to cleave the HA protein (7, 9). These proteases are expressed in the human lung and have the potential to cleave HA *in vivo* (7). TTSPs play a large role in maintaining epithelial homeostasis in epithelial tissues as well as initiating proteolytic cascades and hormone or growth factor activation (10).

There are also a variety of secreted, trypsin-like proteases, such as tryptase and matriptase, which have been shown to cleave HA *in vitro* (11).

This cleavage gives rise to a prefusion state HA, priming the fusion peptide for fusion with the host cell membrane (Figure 1.2) (12). As the early endosome shifts to the late endosome and the pH drops, a conformational change occurs in the HA protein, as (Figure 1.2) (12). The HA protein unfolds, relocating the fusion peptide so that it is no longer buried and can initiate fusion with the host cell membrane (5).

A fusion pore is formed by the release of the segmented genome into the host cell (5). The genome is brought to the nucleus where it can use host cell mechanisms to create the mRNA necessary to start the new influenza virus (1). Once the viral ribo-nucleoproteins (RNP) are exported out of the nucleus, they translocate and are assembled at the apical plasma membrane (1). M2 plays a role in packaging the viral particles while M1 is important in closing and budding off the newly formed influenza particle (1). Before budding from the host cell, NA must cleave off the sialic acid on the viral envelope, preventing aggregation of virus and allowing it to be released from the cell (1, 14).

Influenza Prevention and Treatment

The influenza vaccine is the most effective way to prevent influenza infection. Annual influenza vaccination is recommended for anyone above six months (15). The most widely used seasonal vaccine is the trivalent or quadrivalent inactivated vaccine (16). It is made up of three or four currently circulating influenza strains, two or three influenza A types and one influenza B type (16). The WHO coordinates global surveillance data to determine which three influenza types will be present in the current vaccine (17). The strains are picked relatively early compared to the influenza season, as it requires time to

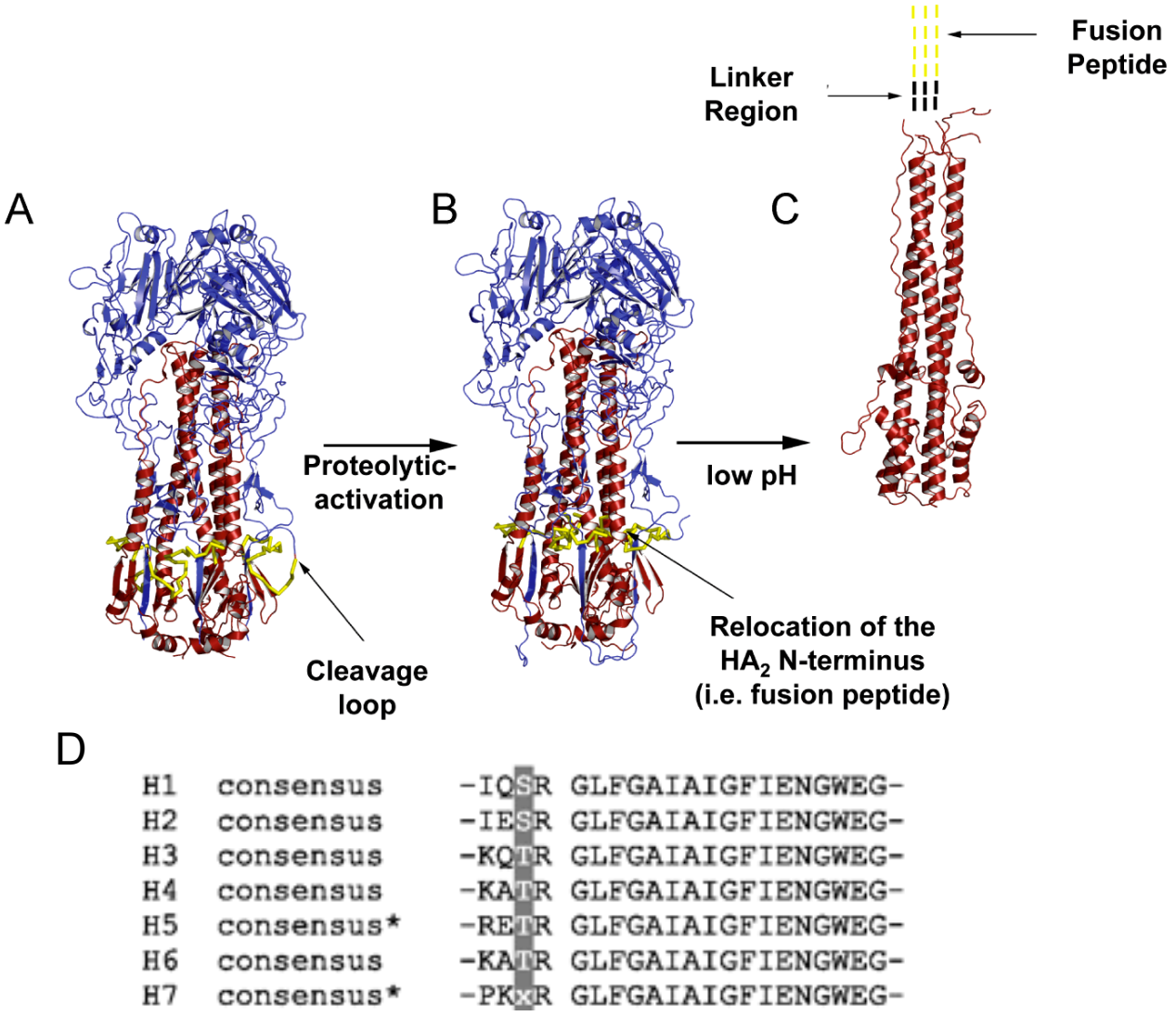


Figure 1.2 (modified from ref. 13) HA conformational change to induce fusion with host cell membrane. HA is cleaved at the cleavage loop by host cell proteases to release fusion peptide (yellow). A low pH induces a conformational change, extending the fusion peptide and allowing for fusion to occur. D) Variety of cleavage loop consensus sequences showing conserved cleavage site (modified from ref. 18)

manufacture the vaccine (16). If no matching strains are chosen for the prevalent influenza types during the influenza season, the vaccine loses its efficacy. Even when strains are matched well, the vaccines do not induce long-lasting antibody titers, resulting in about a 47% effectiveness rate (19).

There are also a variety of antiviral drugs approved by the Food and Drug Administration (FDA) to treat influenza after it has been contracted. Antivirals decrease the time period of infection as well as lessen symptoms that occur because of infection (17). They are mainly effective if taken within 48 hours of infection (17). Originally, there were two classes of anti-influenza drugs available, adamantanes and NA inhibitors (17). The adamantanes include the drugs amantadine and rimantadine and work by blocking the M2 ion channel and interfering with the early stages of viral replication (17). Shortly after adamantanes were discovered, resistant influenza strains began to show up due to changes in the M2 ion channel (20). NA inhibitors include oseltamivir and zanamvir and work by blocking exit of the virus from the infected host cell (17). Although oseltamivir and zanamvir are the current antivirals to treat influenza, there are increasingly more cases of influenza resistant strains (20). Because these drugs target viral proteins, they will always be susceptible to changes in the viral genome (20).

More recently, researchers have begun to look at targeting host cell mechanisms that are used by the virus to replicate (21). Some methods currently being studied are removing the areas of sialidase that are recognized by influenza viruses as well as targeting host signaling pathways that play a role in influenza replication (21). Another way is to use protease inhibitors to limit HA cleavage (21). Recently, peptide-mimetic protease inhibitors of HAT and TMPRSS2 were shown to block viral replication *in vitro* through the inhibition of HA cleavage (22). Also, a serine protease inhibitor, aprotinin, has been studied as an anti-influenza agent. (20) There are already a variety of aprotinin compounds licensed as drugs for post-operative bleeding and pancreatitis (20). However, its characteristics as a serine protease inhibitor made it a good choice to inhibit proteases responsible for HA

cleavage (20). It has been shown to reduce the symptoms of influenza by limiting infection (20).

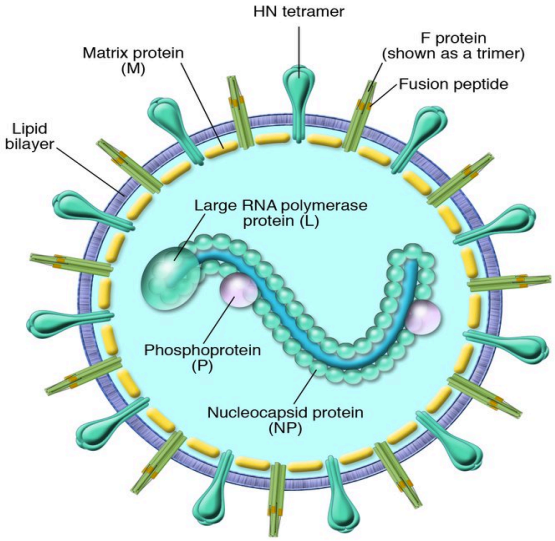
Human Parainfluenza 1 Virus

Human parainfluenza I virus (HPIV-1) causes upper and lower respiratory illness that results in fever, runny nose, and cough (23) More serious symptoms are croup (swelling around the windpipe), bronchitis, and pneumonia (24). HPIVs are a leading cause of acute respiratory infections in children, with HPIV-1 causing about 28,900 hospitalizations annually (24). Transmission can occur through infectious fluids or the inhalation of airborne particles (23). These infections are often reoccurring and only controlled by disinfection of the environment (23). There are currently no vaccines or treatments for HPIV-1 (23).

Human Parainfluenza 1 Structure and Replication

HPIV-1 is an enveloped, non-segmented, negative sense single-stranded RNA virus (25). There are six common structural proteins encoded in the genome (25). There are two surface proteins, hemagglutinin-neuraminidase (HN) and the fusion protein (F) (25). The HN protein is important in viral entry while the F protein regulates the fusion of the virus envelope with the plasma membrane (26). The nucleoprotein (N), phosphoprotein (P), and large (L) proteins form the ribonucleoprotein complex to form its encapsulation (26). Finally, the matrix (M) protein is involved in budding from the plasma membrane of the infected cell (26).

A



B

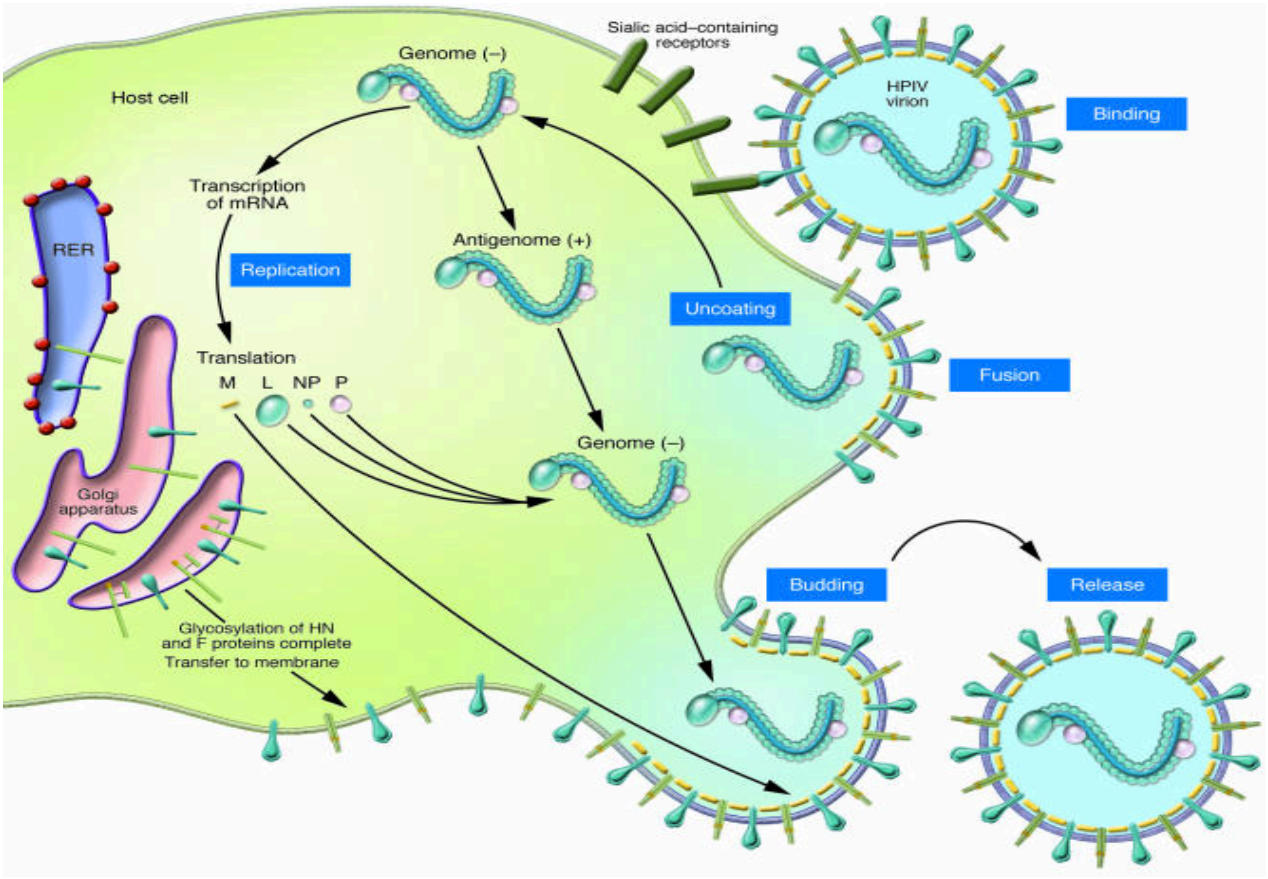


Figure 1.3 (modified from ref. 26) A) Structure of a parainfluenza virus depicting unsegmented viral genome and viral proteins B) HPIV-1 replication pathway: binding to sialic acid, fusion with cell membrane and release of viral genome, protein synthesis, budding and release from cell membrane

Similar to influenza, the HN protein binds to sialic acid when first infecting a cell (26). The HN protein must be present in order for fusion to occur (27). The F protein must then be cleaved by host cell proteases into two subunits so that the fusion peptide can be exposed and inserted into the target cell membrane (27). Then, the HPIV-1 nucleocapsid is released into the cytoplasm of the cell (25). Transcription occurs using the virus-specific RNA-dependent RNA polymerase while cellular mechanisms translate the viral mRNA into viral proteins (25). The matrix protein along with the N and P proteins form the nucleocapsid and then bud off the plasma membrane, releasing a newly formed virus (26).

Hepatocyte Growth Factor Activator Inhibitors

Hepatocyte growth factor activators 1 and 2 (HAI-1 and HAI-2 respectively) are two broad range serine protease inhibitors belonging to the Kunitz family of serine protease inhibitors (28). Both are type 1 transmembrane glycoproteins that contain two extracellular Kunitz-type domains and are closely related (28). The biological role of HAI-1 is thought to be as a potent inhibitor of hepatocyte growth factor activator (HGFA), a blood coagulant that converts hepatocyte growth factor into its active form (29). HAI-1 has also been found as a complex with matriptase in a variety of cells, as seen in Figure 1.4 (30). It is believed to regulate the activity of activated matriptase. Matriptase is a serine protease involved in a variety of proteases and has also been shown to have a significant role in tumor biology (31). HAI-2 is also present in many cells in association with matriptase and is believed to regulate prostasin-dependent matriptase zymogen activation (32). Because of their roles in the regulation of matriptase, HAI-1 and HAI-2 have been investigated as tumor suppressants (33). As a broad range inhibitor, HAI-1 has also been shown to inhibit

a variety of other proteases in the respiratory tract (33). The correlation between proteases that cleave and HA and those that are inhibited by HAI-1 or HAI-2 show shows promise in the use of HAI-2 as an antiviral therapeutic.

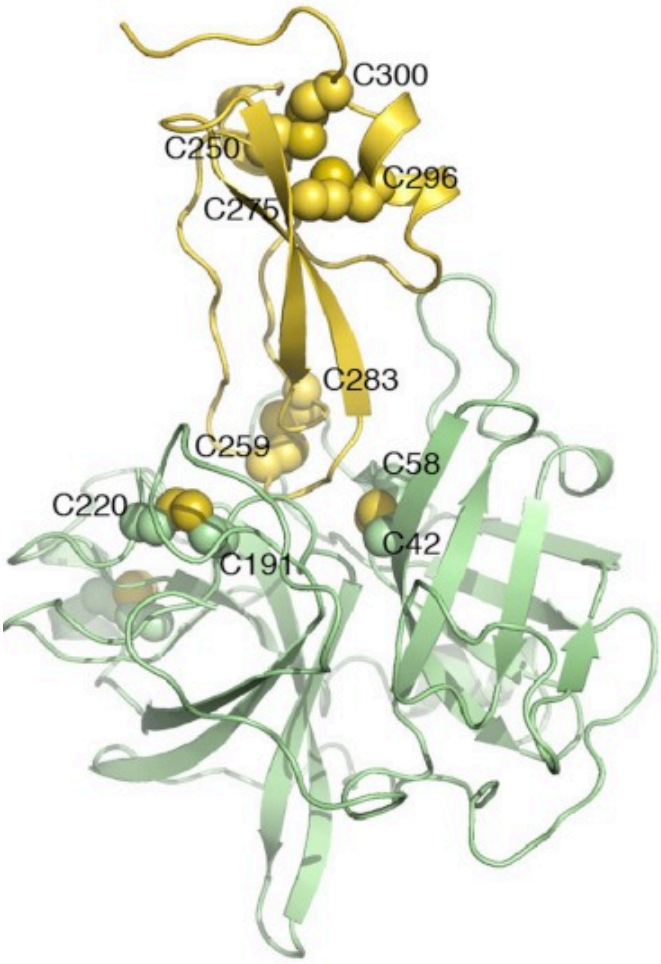


Figure 1.4 (modified from ref. 30) Crystal structure of HAI-1(yellow) bound to matriptase (green)

Methods

HAI-2 Protein Purification

The gene encoding for the ectodomain of mouse HAI-2 (MGC-6479) was obtained from the mammalian gene collection and cloned into a modified pSUMO vector (provided by Holger Sondermann) and named HAI2-pSUMO by Brian Hamilton. HAI2-pSUMO was transformed into *E.* coli, RIL(DE3) (ArticExpress) by Brian Hamilton. The *E. coli* cells transformed with HAI2-pSUMO were grown in 50 mL of Luria broth (LB) containing 35 μg/mL kanamycin at 37°C overnight. Five mL of the culture was used to subculture a 1 liter culture of LB containing 35 μg/mL kanamycin. Once the culture reached an OD of 0.5 to 0.8, it was induced with 0.2M IPTG and incubated overnight at 20°C. The cells were collected by centrifugation and resuspended in lysis buffer (50 mM Tris, 300 mM NaCl, 10 mM imidazole, pH 7.8). The cells were sonicated for 4 minutes at 2 seconds on and 2 seconds off and cell debris was removed by centrifugation. The bacterial lysate was filtered and loaded onto a 7 mL bed volume, Ni^{2+}-NTA agarose resin gravitational column that was pre-equilibrated with five column volumes lysis buffer and subsequently washed with thirty column volumes wash buffer (25 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 7.8). HAI-2 was eluted with two column volumes elution buffer (25 mM Tris, 150 mM NaCl, 200 mM imidazole, pH 7.8) and transferred to a 15-mL Falcon tube. The concentration of HAI-2 was determined by Nanodrop and thrombin was subsequently added to cleave off the His-tag. The elution was incubated overnight at 4°C. The eluted HAI-2 was then loaded onto a Superdex200 size-exclusion column equilibrated with GFP buffer (50 mM Tris, 150 mM NaCl). It was run for 90 mL at 0.4 mL/min. The eluted peaks were tested for HAI-2 by SDS-PAGE. For studies in mice, purified HAI-2 was treated to remove bacterial endotoxins using

a 1 mL Detoxi-Gel Endotoxin Removing Column from Thermo Scientific according to the manufacturer's protocol. The protein concentration was then determined by Nanodrop.

HA peptide cleavage inhibition assay

Peptides were designed to mimic the consensus cleavage site region of the HA of the H1 (IPSIQSRGLF) and H3 (VPEKQTRGLF) subtypes. As a FRET pair, MCA (7-methoxycoumarin-4-yl acetyl) was added to the N-terminus, and DNP (N-2,4-dinitrophenyl) was added to the C-terminus (RS Synthesis). HAI-2, trypsin and each peptide were diluted in buffer B, producing a final concentration of 0-1 μM HAI-2, 0.8 nM trypsin, and 100 μM peptide. The reaction was immediately carried out at 37°C and monitored for cleavage by the change in fluorescence at 390 nm (SpectraMax GeminiXS, Molecular Devices). The IC_{50} value denotes the HAI-2 concentration at which trypsin was inhibited by 50%, as compared to the uninhibited control. The initial rate at each HAI-2 concentration was both plotted and fitted using Origin software (OriginLab Corp.) to determine the IC_{50} values.

HA cleavage inhibition by Western blot

293T cells were transfected with 1 μg of each HA-expressing plasmid using Lipofectamine 2000 (Invitrogen) for 12 hours at 37°C. Three μg/ml trypsin along with a concentration range of HAI-2 (0-1 μM) were pre-incubated in buffer A for 10 min at 37°C, the cells washed with phosphate buffered saline (PBS) and treated with each protease-inhibitor mixture for 45 min at 37°C. The cells were then processed by cell surface biotinylation in preparation for western blot analysis. Inhibition of HA cleavage was assessed by western blot using anti-A/PR/8/34 (H1) and anti-A/Hong Kong/1/68 (H3)

antibodies (NIAID Biodefense & Emerging Infections Research Resource Repository). Western blot images were taken by FujiFilm LAS-3000. The pixel intensity of the individual band was measured by Image J, and relative cleavage efficiencies were calculated by the following equation: (HA_2/HA_0+HA_2) x100%.

Inhibition of influenza infection in cell culture

To measure infection of individual cells, non-cleaved A/PR8/34 was generated by incubation of 1 pfu/cell egg-derived virus with 293T cells in a 6 cm dish containing RPMI for 1 hr at 37°C. 293T cells were chosen due to their lack of endogenous proteases capable of cleaving HA. The non-cleaved virus was incubated with the inhibitor-protease mixture described above for 20 min at 37°C. Cleavage of HA was terminated by the addition of 5 μg of trypsin inhibitor (EMD Chemicals). MDCK cells were grown in 24-well plates containing glass cover slips and infected with the virus at each HAI-2 concentration and incubated for 5 hr at 37°C. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, washed with PBS and assessed for infection by immunofluorescence using a polyclonal anti-nucleoprotein antibody coupled followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody. The cell nuclei were stained with Hoechst 33258 or DAPI. Cells were images on a Nikon E600 epifluorescence microscope using a 20X objective.

To measure virus replication and spread, egg-derived influenza A/PR8/34 (containing cleaved HA) was used to infect MDCK cells at a low multiplicity of infection (approximately 0.01 pfu/cell) in 24-well plates. Trypsin was included in the media to allow virus spread, and samples were treated with each HAI-2 concentration, or were untreated. Media was collected at 48 hr, remaining trypsin activity was blocked by the addition of 5 μg

of trypsin inhibitor, and supernatant containing virus was transferred to a 96-well plates for virus quantification by hemagglutination assay.

HAI-2 Effect on VSV

MDCK cells were infected with vesicular stomatitis virus (VSV) in the presence or absence of HAI-2 and 1 μg/mL trypsin for five hours. The concentrations of HAI-2 used were: 10 nM, 50, Nm, 150 nM, 5000 nM, or 1000 nM. Infection was then monitored by immunofluorescence microscopy. The cells were fixed with 4% PFA and permeabilized with 0.5% Triton in PBS. The cells were blocked with 10% FBS in PBS. The primary antibody used was mouse anti-VSV glycoprotein (AbCam) at a 1:500 dilution. The secondary antibody used was anti-mouse Alexafluor488 (Invitrogen) at a 1:1000 dilution. The nuclei were stained with DAPI. Images were taken with a Nikon Eclipse e600 microscope using a 20x objective.

Inhibition of HPIV-1 infection in cell culture

LLC-MK2 cells were infected with HPIV-1 at low multiplicity of infection. The virus was incubated for one week at 37°C in the presence of 1 μg/mL exogenous trypsin. Cells were treated with a range of HAI-2 concentrations: 10 nM, 50 nM, 150 nM, 500 nM, and 1000 nM. Trypsin was added every 48 hours to replenish the supply. After seven days, the cells were fixed with 4% PFA and permeabilized with 0.5% Triton. Immunofluorescence microscopy was used to look at spread of infection using a Nikon Eclipse microscope using a 20x objective. Mouse anti-parainfluenza virus type 1 antibody conjugated to FITC was used to visualize the infection. The nuclei were stained with DAPI.

HAI-2 Toxicity Test

Eight-week-old balb-c mice were administered varying concentrations of HAI-2 or PBS. There were four groups of mice, each with three mice in each group. Each group was administered 0.5 mg/kg, 0.75 mg/kg, 2.5 mg/kg HAI-2 or PBS only. The doses were administered intranasally under anesthesia (isoflurane). Each group was housed in a different cage depending on treatment. The mice were given 50 μL doses of PBS or HAI-2 every 12 hr for three days. The mice were monitored twice daily for weight loss.

Influenza PR8 Lethality Test

Eight-week old balb-c mice were infected with mouse-adapted influenza, A/PR/8/34 (PR8) ranging from 1 foci fluorescent unit (ffu) to 1000 ffu or PBS. There were six groups of mice with three mice in each group. The mice were infected via intranasal administration under anesthesia (isoflurane) with the varying concentrations diluted in 50 μL PBS. Each group was housed in a different cage depending on their infection dose. The mice were infected on Day 1 and their weight was monitored daily over the course of twelve days. Animals showing weight loss greater than 20% of their initial weight were euthanized.

PR8 HAI-2 Challenge

8-week-old female balb-c mice were inoculated under mild anesthesia (isoflurane). The inoculums were administered intranasally, with half of total volume per nostril. Treatment was performed at time 0 with 0.75 mg/kg HAI-2 diluted in 50 μL PBS. After four hours, infection was performed with a 50 μL solution containing 100 ffu of influenza virus (A/PR/8/34). Subsequent HAI-2 administrations were performed at 12-hour intervals during the course of five days.

The experiment was performed with five mice in each group, with animals housed in different cages depending on treatment. The four groups were as follows: 1) PBS only 2) HAI-2 only 3) PR8 only 4) HAI-2/PR8. Mice were monitored twice daily for clinical signs, such as scruffiness and body weight loss. Animals showing weight loss higher than 30% of initial weight were euthanized. Lung tissue was removed post-mortem and utilized for viral titer determination by foci fluorescent assay.

Results

HAI-2 Protein Purification

HAI-2 was first purified by a Ni^{2+} agarose column since the HAI-2 protein had been designed with a His-tag attached to it. However, as can be seen in Lane 5, there are still a variety of other bands present after this purification process (Figure 4.1). The size exclusion column was then used to eliminate these other bands as well as the thrombin that was used to cleave the His-tag. Two peaks were present in the fast protein liquid chromatography (FPLC) trace. As can be seen in Lane 6, the first peak contained the purified HAI-2 at 21 kDa. However, it was still not completely purified, as two other bands were also present. The lower band could be degraded HAI-2. Both bands could also be a result of nonspecific binding. In the second peak, there was a band at about 75 kDa, showing the thrombin was effectively removed from the HAI-2 sample. HAI-2 was purified from the *E. coli* resulting in a yield of 0.4 mg/L. In order to further purify the sample in the future, the purified HAI-2 could be run through the Ni^{2+} agarose column, allowing the cleaved HAI-2 to elute while allowing for anything nonspecific to bind to the column.

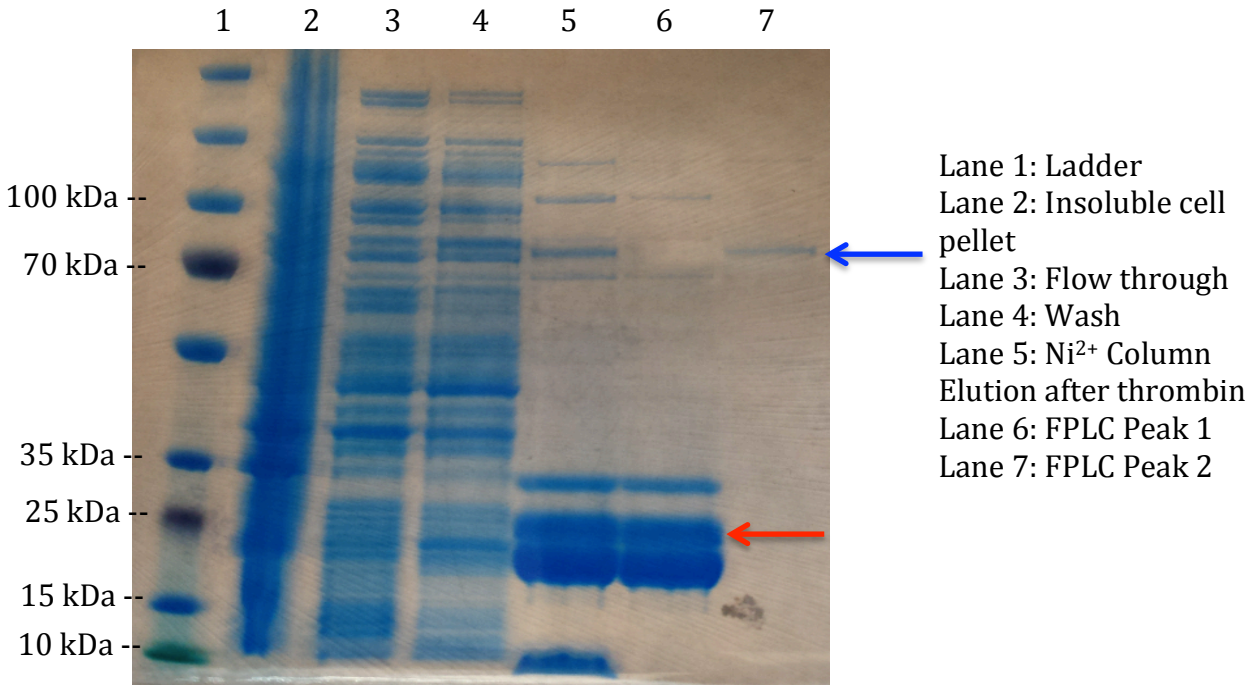


Figure 4.1 SDS-PAGE gel depicting different stages of HAI-2 purification process, as labeled by key. Purified HAI-2 (red arrow) and removed thrombin (blue arrow)

In Vitro HAI-2 Characterization

HAI-2-mediated inhibition of peptide mimics of H1N1 and H3N2 influenza

Inhibition of peptide mimics of the HA cleavage site by HAI-2 was first investigated, using the established activity of trypsin as a surrogate of *in vivo* trypsin-like proteases (34). HAI-2 activity was competed with HA cleavage site peptide mimics of both the human-adapted H1 and H3 subtypes (A/PR/8/34 and A/Aichi/2/68 respectively). The peptide sequences were designed based on the consensus amino acid sequence of cleavage site region of each subtype. Competition of HAI-2 and each peptide with trypsin resulted in an IC_{50} value of 22.4+/-10nM for the H1 peptide mimic and 89.9+/-6nM for the H3 peptide mimic (Fig. 4.2 A and B). While HAI-2 was slightly more active against cleavage of the H1 subtype peptide mimic, potent inhibition was observed against both peptide substrates. Work done by Brian Hamilton.

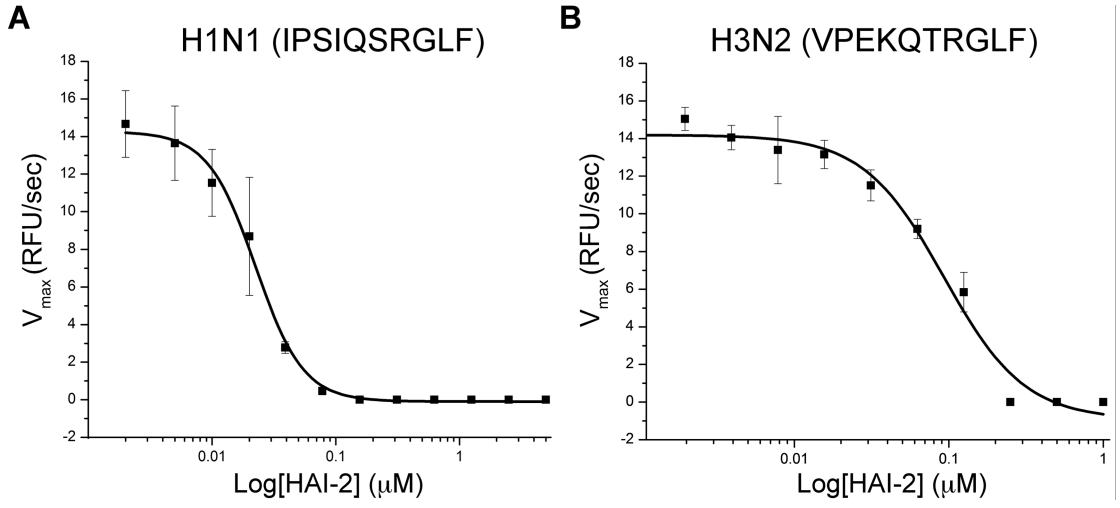


Figure 4.2 Graphs of HAI-2 inhibition of trypsin cleavage of A) the H1 subtype cleavage site peptide mimic and B) the H3 subtype cleavage site peptide mimic. Peptide sequences indicated in titles of respective graphs. Experiment performed by Brian Hamilton.

Inhibition by HAI-2 of influenza H1N1 and H3N2 HA cleavage

To determine whether HAI-2 has the ability to inhibit HA cleavage in cell culture, HAI-2 at a concentration range of 0-1 μM was incubated with trypsin, and subsequently incubated with HA-expressing cells. Inhibition of HA cleavage was assessed by western blot analysis, where HA2 bands are not detected well with the anti-sera used, which is selective for the HA1 subunit. Effective inhibition of HA cleavage was observed for both the H1 and H3 subtypes (A/PR/8/34 and A/Aichi/2/68 respectively), where cleavage was virtually abolished at an HAI-2 concentration of 150 nM, and greatly reduced at 50 nM HAI-2 (Fig. 4.3 A and B). Thus, cleavage of the HA from both of the currently circulating human-adapted subtypes is potently inhibited by HAI-2. Work done by Brian Hamilton.

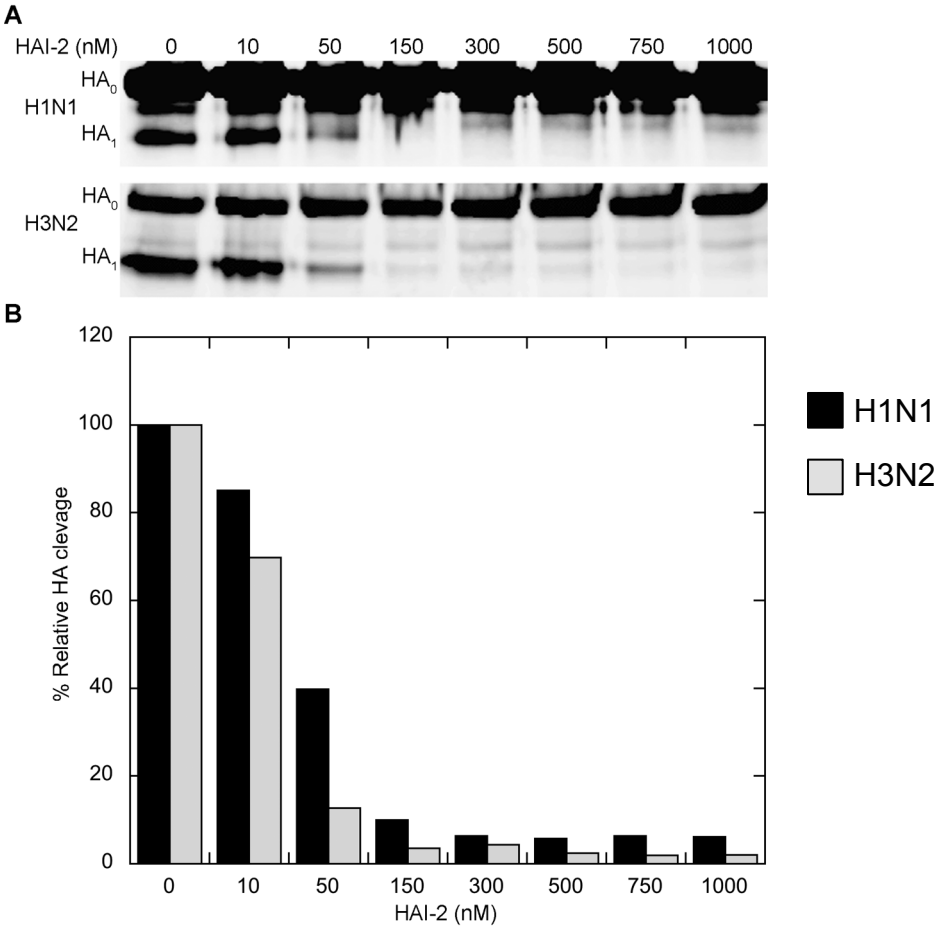


Figure 3.3 A) Western blot analysis of HAI-2 inhibition of H1N1 HA and H3N2 HA cleavage by trypsin B) Quantification of the percent HA cleavage from A) using densitometry. Experiment performed by Brian Hamilton

Inhibition of influenza virus infection by HAI-2

As an additional measure of inhibition in cell culture, HAI-2 activity on infectious virus particles was determined, both directly at the level of virus entry, as well as at the level of virus spread. To examine virus entry directly, non-cleaved, inactive influenza A/PR/8/34 (H1N1) was generated by a single round of replication in mammalian cells that are incapable of cleaving HA. Inhibition of viral activation was assessed by treatment of the virus with trypsin and various concentrations of HAI-2, followed by immunofluorescence staining of the viral nucleoprotein in infected cells. Similar to inhibition of HA cleavage observed by western blot analysis, effective inhibition of infection of influenza A/PR/8/34 infection was observed by treatment with HAI-2 (Fig. 4.4 A). Work done by Brian Hamilton.

To examine the effects of HAI-2 on influenza virus propagation and spread, influenza A/PR/8/34 (H1N1) was produced in embryonated chicken eggs, yielding virions with cleaved and active HA. These viruses were used to infect MDCK cells at a low multiplicity of infection, and cells were incubated for 48 hr to allow virus replication and spread. The $TCID_{50}$ was attempted, but infected cells were difficult to discern. Media was then harvested and virus was quantified by hemagglutination assay (Fig. 4.4 B). Potent inhibition of influenza A/PR/8/34 infection, in a dose-dependent manner, was observed by treatment with HAI-2. Overall, HAI-2 effectively inhibited influenza virus infection in cell culture. Work done by Yoko Yoda.

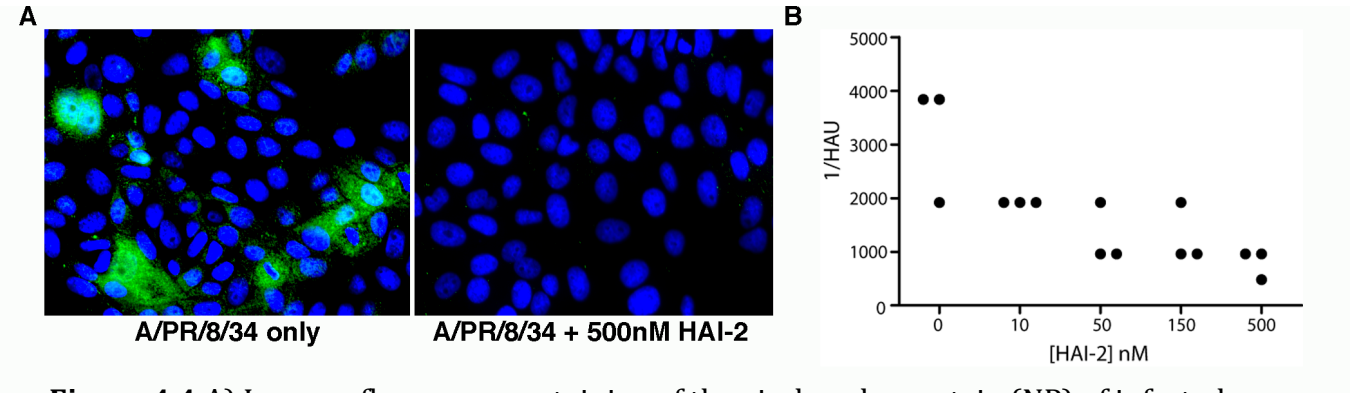


Figure 4.4 A) Immunofluorescence staining of the viral nucleoprotein (NP) of infected cells after trypsin treatment with 500 nM HAI-2, and no protease treatment (mock). The viral NP was labeled with Alexa fluor 488 (green) and the nucleus was stained with DAPI (blue). B) MDCK cells were infected with a low MOI of influenza virus, and infection was allowed to spread for 48h in the presence of representative amounts of HAI-2. Virus yield was measured by hemagglutination (HA) assay in triplicate, and individual HA units (HAU) in each condition plotted. Experiments performed by Brian Hamilton and Yoko Yoda.

Effects of HAI-2 on other enveloped viruses

As a control, we assessed the effect of HAI-2 on the entry of an unrelated virus (VSV) that contained a glycoprotein (G) that is not activated by proteolytic cleavage (35). An incubation period of 5 hours allows enough time for VSV to infect cells, but not long enough for it to replicate and spread. As the concentration of HAI-2 increases, VSV still has the ability to infect cells (Figure 4.5 A). We also assessed the effect of HAI-2 on the entry of a related virus (HPIV-1) that contained a fusion protein that is believed to be activated by similar proteases to influenza HA. As with influenza infection, HAI-2 treatment inhibited HPIV-1 infection in a dose-dependent manner (Figure 4.5 B).

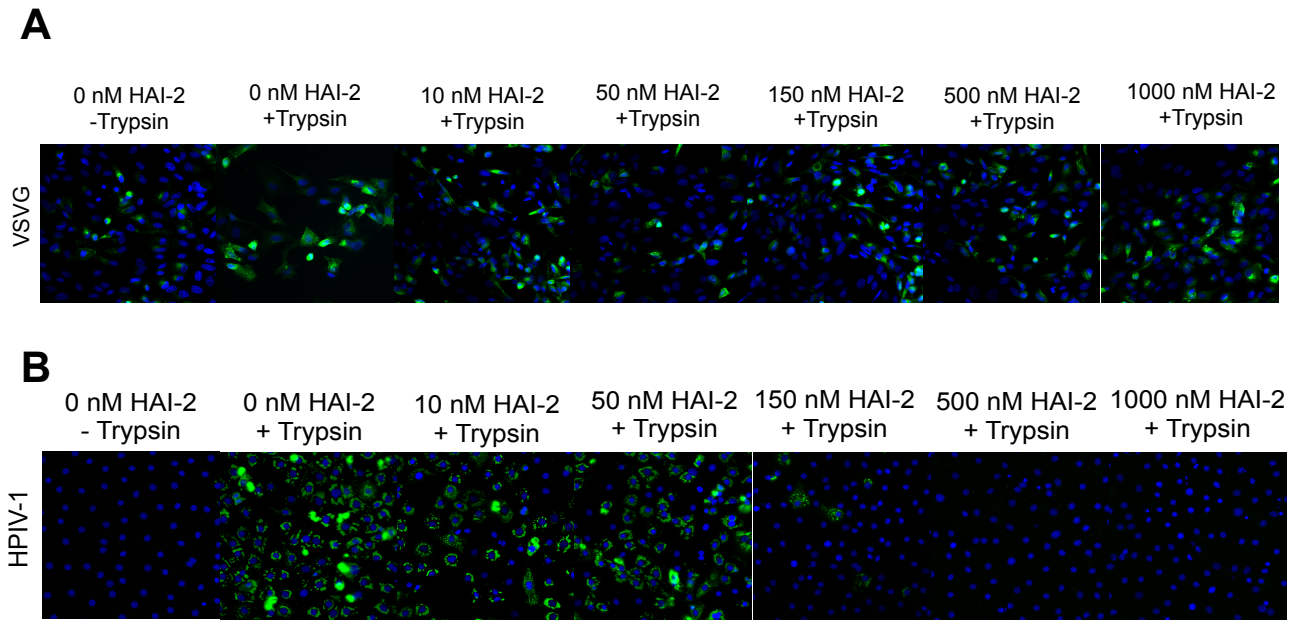


Figure 3.5 A) Immunofluorescence staining of the VSV glycoprotein (G) in infected cells after trypsin treatment with representative amounts of HAI-2, and no protease treatment (mock). The viral G protein was labeled with Alexa fluor 488 (green) and the nucleus was stained with DAPI (blue). B) Immunofluorescence staining of the HPIV-1-infected cells after trypsin treatment with representative amounts of HAI-2, and no protease treatment (mock). HPIV-1 is labeled with FITC (green) and the nucleus was stained with DAPI (blue).

In Vivo Characterization of HAI-2 using a Mouse Model

HAI-2 Toxicity Text

To first examine any potential toxicology problems with HAI-2, balb/c mice were exposed intranasally to HAI2 at doses of 2.5 mg/kg, 0.75 mg/kg, and 0.5 mg/kg. Body weight and clinical signs were monitored for 4 days and mice were then euthanized. PBS was used as a control to ensure delivery methods were not harmful to the mice. The administration of HAI-2 at different doses did not harm the mice, as no significant weight loss or clinical signs were shown (Figure 4.6).

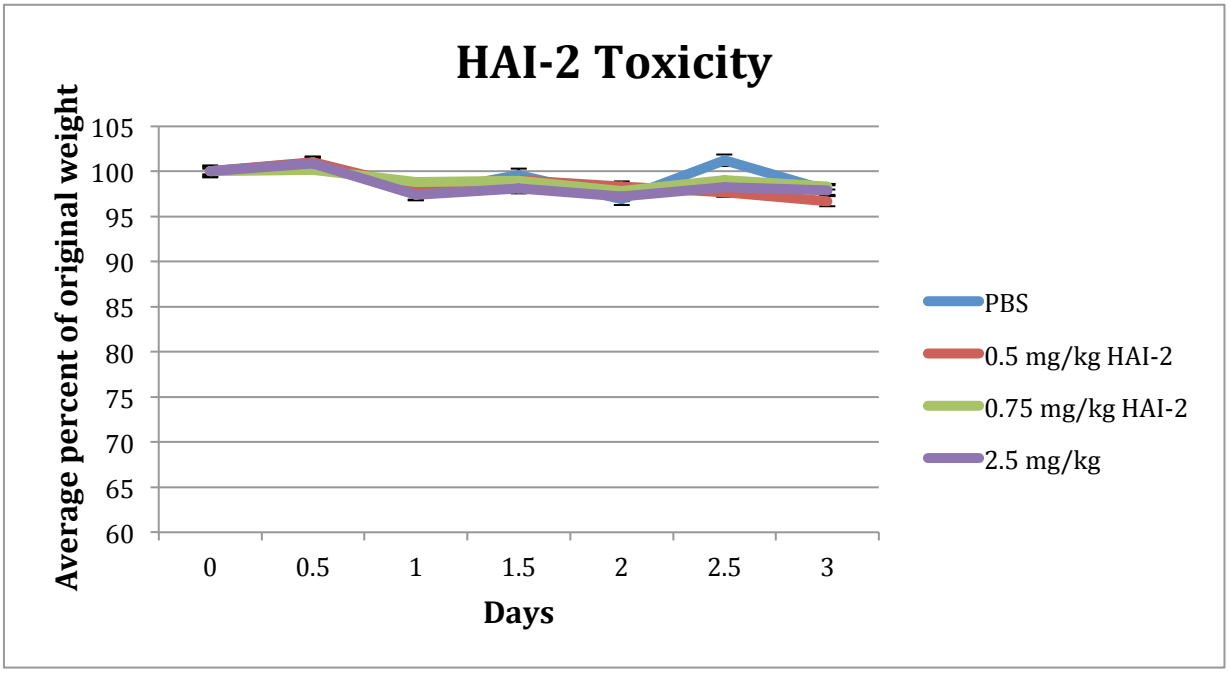


Figure 4.6 Graph depicting average percent of original weight loss from each group of mice vs. days of experiment. Mice were treated with 0.5 mg/kg, 0.75 mg/kg, or 2.5 mg/kg HAI-2 or PBS intranasally.

Influenza A/PR/8/34 Lethality Test

To determine the lowest lethal dose of A/PR/8/34, balb/c mice were exposed intranasally to A/PR/8/34 at 1 ffu, 10 ffu, 100 ffu, 1000 ffu, or 10000 ffu. Body weight and clinical signs were monitored for 12 days and mice were euthanized upon losing thirty percent of their original weight. PBS was used as a control to ensure delivery methods were not harmful to the mice. Mice given any dose above 10 ffu quickly began to lose weight on the third day of the experiment. At 10 ffu, mice lost weight originally but were able to recover. 100 ffu was the lowest lethal dose of A/PR/8/34 determined (Figure 4.7).

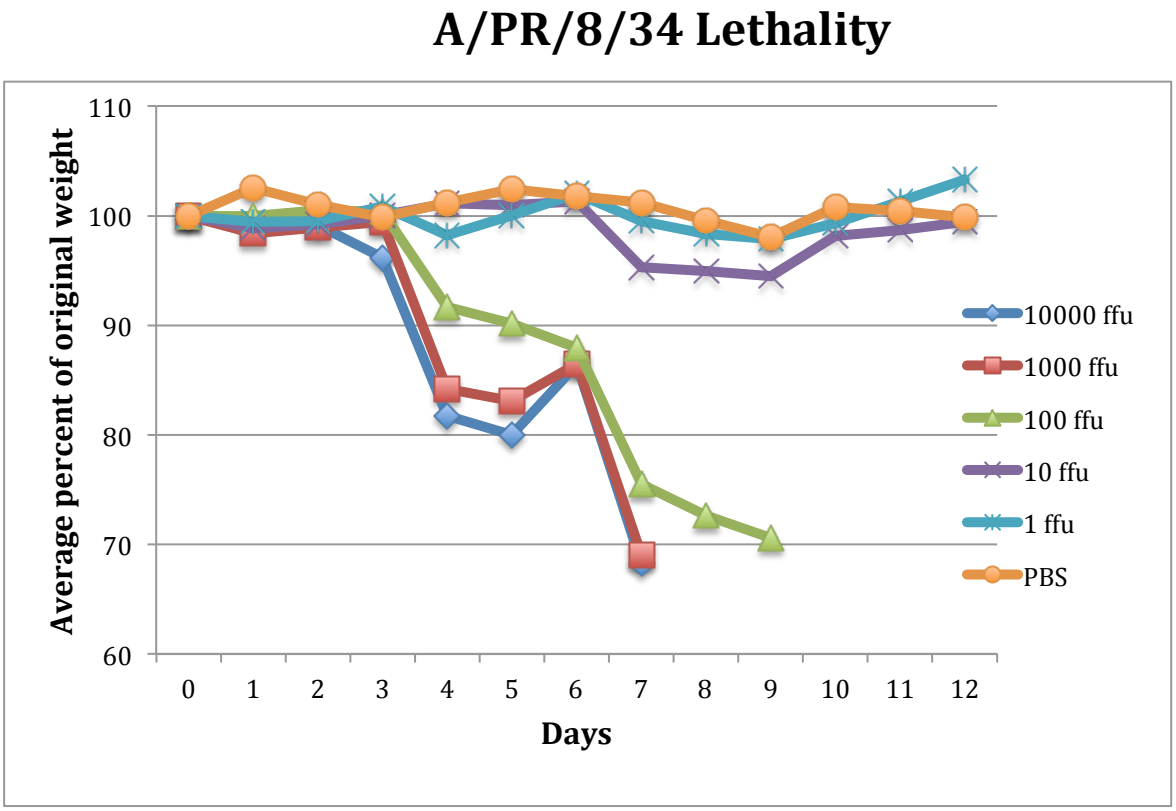


Figure 4.7 Graph depicting average percent of original weight loss from each group of mice vs. days of experiment. Mice were treated with varying doses of A/PR/8/34.

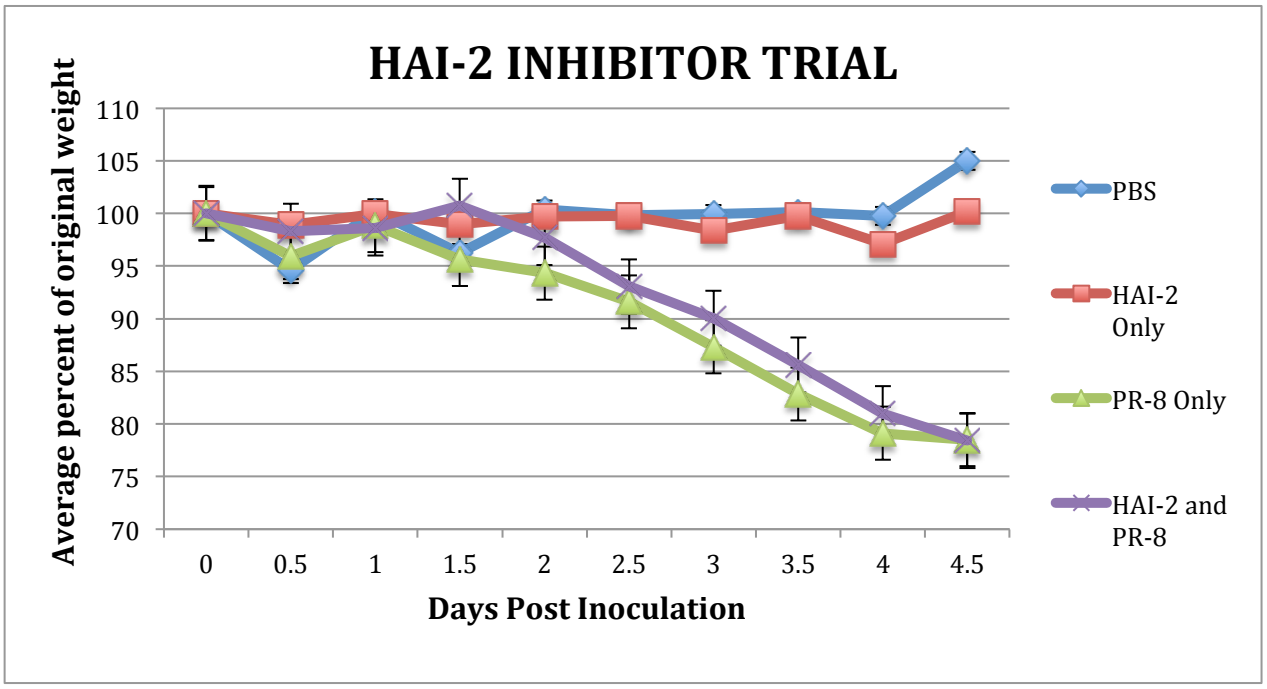
Inhibition of influenza virus infection by HAI-2 in a mouse model

There are two major ways mice display symptoms when infected with influenza: weight loss and survival. Both of these symptoms were monitored during the course of the experiment. To determine the inhibition effects of HAI-2 against influenza infection, mice were given an initial dose of 0.75 mg/kg HAI-2 and then challenged by 100 ffu A/PR/8/34 intranasally. HAI-2 was administered intranasally every 12 hours through the course of the experiment. Mice were weighed and monitored twice daily for 5 days. Mice treated with HAI-2 as well as those only infected with influenza lost similar amounts of weight during the experiment (Figure 3.8 A). Although there was a slight delay in weight loss by the HAI-2 treated mice, there was no significant difference in the amount of weight lost in the mice treated with HAI-2 (unpaired p-test, p value = 0.5932).

When determining survival, mice that had spontaneously died during the course of the experiment were plotted on a survival graph (Figure 3.8 B). Spontaneous death was defined by mice that were not euthanized but rather, were found dead while being monitored. On the fourth day, one influenza-only mouse was found dead, while on the second day two more influenza-only mice were found dead and one mouse treated with HAI-2 was found dead. Although the slight delay is still present when looking at mouse survival, there was no significant difference (Mandel-Cox, p value = 0.5439).

Post-experiment, mouse lungs were harvested to determine viral titer present in the lung tissue. Mice infected with influenza had an 8.3×10^2 ffu/g viral titer while those treated with HAI-2 had a 7.0×10^2 ffu/g viral titer. There was a significant decrease in the viral titer of the mice treated with HAI-2 (unpaired t-test, p value = 0.0001).

A



B

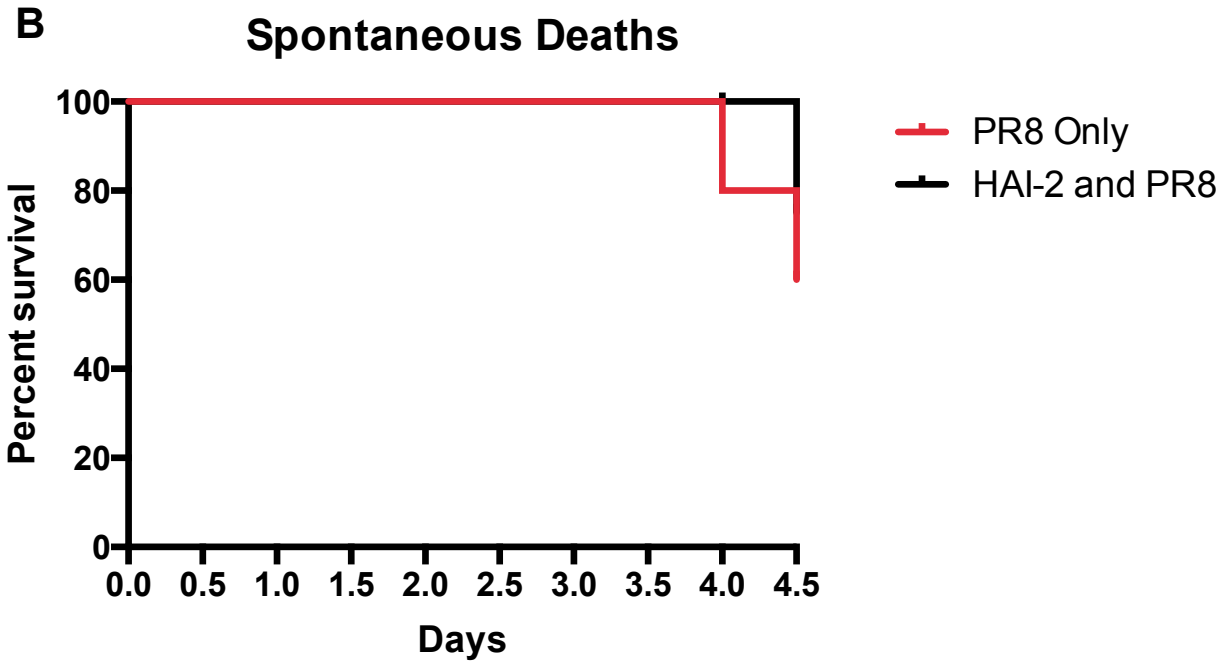


Figure 3.8 A) Graph depicting average percent of original weight loss from each group of mice vs. days of experiment. Mice were given an initial treatment of HAI-2 and then challenged with A/PR/8/34. HAI-2 treatment was then given every 12 hours. B) Survival of mice during experiment.

Discussion

Despite availability of vaccination and antiviral therapeutics, influenza still remains a significant concern for public health. New approaches to treating influenza need to be explored to combat newly emerging drug-resistant influenza strains. Targeting host functions rather than viral proteins may be more effective, due to the decreased likelihood for the emergence of resistant influenza strains. The serine protease inhibitor, HAI-2, is highly attractive anti-influenza candidate due to the high correlation between the proteases that HAI-2 inhibits and the proteases shown to activate HA. We therefore investigated whether HAI-2 has the ability to inhibit HA cleavage, using trypsin as an established model protease for influenza infection in cell culture.

HAI-2 was successfully purified for use in experiments with a yield of 0.4 mg/L. Despite the nonspecific bands present, HAI-2 showed activity *in vitro* and *in vivo*. HAI-2 showed potent inhibition of trypsin cleavage *in vitro*, as determined by the nM IC_{50} values. HAI-2 was also able to inhibit infection and spread of both A/PR/8/34 and HPIV-1 in cell culture starting at 500 nM concentration. Furthermore, HAI-2 did not affect infection of an unrelated virus (VSV). This shows that a high concentration of HAI-2 does not inhibit the ability for unrelated viruses to enter the cell, but is specific towards viruses that require trypsin or trypsin-like proteases to infect host cells.

An influenza mouse model was already established in the lab, allowing for the effects of HAI-2 inhibition of influenza to be looked at *in vivo*. The two major ways mice display symptoms when infected with influenza are weight loss and survival. HAI-2 treatment delivered intranasally showed no toxicity when delivered to mice. Determining the A/PR/8/34 viral titer to use showed that this mouse-adapted influenza is very effective

against mice. Even a low 100 ffu dose was enough to be a lethal dose. Mice infected with A/PR/8/34 and treated with HAI-2 showed no significant difference in weight loss or survival. Mice treated with HAI-2 may not have shown a significant difference in weight loss or survival because there was still a high enough A/PR/8/34 viral titer to stop recovery from occurring. However, viral titer from lung tissue showed a significant decrease in the mice treated with HAI-2, showing that HAI-2 can act on the trypsin-like proteases present in the respiratory tract *in vivo*. Thus, HAI-2 has the potential to be a viable treatment to circumvent influenza infection. Although a comprehensive evaluation of the toxicity and effects of HAI-2 in experimental models more relevant to humans has not been done yet, our findings suggest it can be a viable candidate for further drug development. The inhibition of HPIV-1 by HAI-2 also suggests that it can target additional respiratory viruses with similar entry mechanisms to influenza.

Conclusion

Current influenza treatments are quickly becoming outdated as more influenza strains become resistant to them. A new method for treating influenza needs to be researched that does not focus on targeting influenza proteins, so as to circumvent the changing viral genome. The protease inhibitor, HAI-2, is a promising agent to use as an antiviral to inhibit host proteases and prevent entry of influenza and other similar viruses into host cells. Here, we successfully purified HAI-2 for use in experiments. We showed that HAI-2 does not have any adverse effects on cells, even at high concentrations. HAI-2 is able to inhibit HA cleavage by trypsin in cells. HAI-2 was also able to inhibit the spread of influenza as well as HPIV-1 *in vitro*. Mice infected with PR8 and treated with HAI-2 showed a very modest delay in weight loss and death. HAI-2 was likely able to inhibit host mouse proteases and decrease the replication of PR8 in the mice, as shown by the viral titers from lung tissue. HAI-2 seems to be a promising candidate for an antiviral to treat influenza and other viruses with similar entry mechanisms.

Future Directions

There are several areas to investigate for future studies involving HAI-2. It was shown that HAI-2 decreased the viral titer in mouse lungs, but little recovery was shown while the mice were alive. By utilizing a lower viral titer of A/PR/8/34 and a higher concentration of HAI-2, treated mice could show less weight loss. Also, by delivering the HAI-2 treatment by an aerosol method, rather than intranasally, the amount of HAI-2 reaching the lungs can be more controlled. Since mice have few symptoms of influenza infection, further studies can use ferrets as a model organism. Ferrets have similar symptoms to humans and HAI-2 can be explored in a greater context. The inhibition of HPIV-1 by HAI-2 suggests HAI-2 has the ability to inhibit a variety of respiratory viruses. Other viruses, such as influenza B, can be screened for inhibition by HAI-2. Finally, protein engineering of HAI-2 could be done to try and produce a more effective version of the protein for use as an antiviral therapeutic.

Works Cited

1. Wright, Peter F., Neumann, G., Kawaoka, Y. Orthomyxoviruses. In: Fields Virology sixth edition, Knipe DM, Howley, PM, eds, Lippincott, Philadelphia 2007, 1691-1739.
2. Karve, S., Misurski, D., Herrera-Taracena, G., and Davis, K. L. (2013) Annual all-cause healthcare costs among influenza patients with and without influenza-related complications: analysis of a United States managed care database. *Applied health economics and health policy 11*, 119–28.
3. Webster, R. G., and Govorkova, E. A. (2014) Continuing challenges in influenza. *Annals of the New York Academy of Sciences*.
4. Taubenberger, J., and Kash, J. (2010) Influenza virus evolution, host adaptation, and pandemic formation. *Cell host & microbe 7*, 440–51.
5. Skehel, J. J., and Wiley, D. C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annual review of biochemistry69*, 531–69.
6. Horimoto T., *et al.* Influenza: lessons from past pandemics, warinings from current incidents. (2005) *Nature Reviews Microbiology 3,* 592.
7. Hamilton, B.S., Whittaker, G., Daniel, S. (2012) Influenza Virus-Mediated Membrane Fusion: Determinants of Hemagglutinin Fusogenic Activity and Experimental Approaches for Assessing Virus Fusion. *Viruses 4(7),* 1144-1168.
8. Edinger, T., Pohl, M., and Stertz, S. (2013) Entry of influenza A virus: host factors and antiviral targets. *Journal of General Virology 95*, 263277.
9. Bottcher, E., Matrosovich, T., Beyerle, M., Klenk, H., Garten, W., and Matrosovich, M. (2006) Proteolytic Activation of Influenza Viruses by Serine Proteases TMPRSS2 and HAT from Human Airway Epithelium. *Journal of Virology 80*, 98969898.
10. Bugge, T. H., Antailis T. M., Wu, Q. (2009) Type II Transmembrane Serine Proteases. *J Biol Chem. 284*35),* 23177-23181.
11. Hamilton B. S., Gludish D. W. J., Whittaker, G. R. (2012) Clevage Activtion of the Human-Adapted Influenza Virus Subtypes by Matriptase Reveals Both Subtype- and Strain-Specificity. *Journal of Virology, 86,* 10579-20586.
12. Ni, F., Chen, X., Shen, J., and Wang, Q. (2014) Structural insights into the membrane fusion mechanism mediated by influenza virus hemagglutinin. *Biochemistry 53*, 846–54
13. Galloway, S., Reed, M., Russell, C., and Steinhauer, D. (2013) Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. *PLoS pathogens 9*, e1003151.
14. Huang, I.-C., Li, W., Sui, J., Marasco, W., Choe, H., and Farzan, M. (2008) Influenza A virus neuraminidase limits viral superinfection. *Journal of virology 82*, 4834–43.
15. Uyeki, T. M. (2014) Preventing and controlling influenza with available interventions. *The New England journal of medicine 370*, 789–91.
16. Wong, S.-S. S., and Webby, R. J. (2013) Traditional and new influenza vaccines. *Clinical microbiology reviews 26*, 476–92.
17. Hurt, A. C., Selleck, P., Komadina, N., Shaw, R., Brown, L., and Barr, I. G. (2007) Susceptibility of highly pathogenic A(H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes. *Antiviral research 73*, 228–31.

18. Sun X., Tse L., Ferguson D., Whittaker G. (2010) Modifications ot the Hemagglutinin Cleavage Site Control the Virulence of a Neurotropic H1N1 Influenza Virus. *J. Virol 84,* 8683-8690.
19. Ohmit, S., Thompson, M., Petrie, J., Thaker, S., Jackson, M., Belongia, E., Zimmerman, R., Gaglani, M., Lamerato, L., Spencer, S., Jackson, L., Meece, J., Nowalk, M., Song, J., Zervos, M., Cheng, P.-Y., Rinaldo, C., Clipper, L., Shay, D., Piedra, P., and Monto, A. (2014) Influenza vaccine effectiveness in the 2011-2012 season: protection against each circulating virus and the effect of prior vaccination on estimates. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 58*, 319–27.
20. Zhirnov, O., Klenk, H., and Wright, P. (2011) Aprotinin and similar protease inhibitors as drugs against influenza. *Antiviral research 92*, 27–36.
21. Lee, S., and Yen, H.-L. (2012) Targeting the host or the virus: current and novel concepts for antiviral approaches against influenza virus infection. *Antiviral research96*, 391–404.
22. Bottcher E., Stein D., Klenk H., Garten W. (2011) Inhibition of influenza virus infection in human airway cell cultures by an antisense peptide-conjugated morpholino oligomer targeting the hemagglutinin-activating protease TMPRSS2. *J Virol 85,* 1554-1562.
23. Karron, Ruth A., Collins, Peter L. Parainfluenzas In: Fields Virology fifth edition, Knipe DM, Howley, PM, eds, Lippincott, Philadelphia 2007, 1497-1520.
24. Burke, C., Bridges, O., Brown, S., Rahija, R., and Russell, C. (2013) Mode of Parainfluenza Virus Transmission Determines the Dynamics of Primary Infection and Protection from Reinfection. *PLoS Pathogens 9*, e1003786.
25. Henrickson, K. (2003) Parainfluenza Viruses.*Clinical Microbiology Reviews*.
26. Zuckerman, J. Arie. (2009) Principles and Practice of Clinical Virology. In: John Wiley & Sons. 6th Ed. 410-419.
27. Moscona, A. (2005) Entry of parainfluenza virus into cells as a target for interrupting childhood respiratory disease. *Journal of Clinical Investigation 115*, 1688–1698.
28. Szabo, R., Hobson J., List K., Molinolo A., Lin C., Bugge TH. (2008) Potent inhibition and global co-localization implicate the transmembrane Kunitz-type serine protease inhibitor hepatocyte growth factor activator inhibitor-2 in the regulation of epithelial matriptase activity. *J Biol Chem 283 (43),* 29495-29504.
29. Eigenbrot C., Ganesan R., Kirchhofer D. (2010) Hepatocyte growth factor activator (HGFA): molecular structure and interactions with HGFA inhibitor-1 (HAI-1). *FEBS J*, 2215-2222.
30. Zhao, B., Yuan, C., Li, R., Qu, D., Huang, M., and Ngo, J. (2013) Crystal structures of matriptase in complex with its inhibitor hepatocyte growth factor activator inhibitor-1. *The Journal of biological chemistry 288*, 11155–64.
31. Friis S., Sales KU., Schafer JM., Vogel LK., Kataoka H., Bugge TH., (2014) The protease inhibitor HAI-2, but not HAI-1, regulates matriptase activation and shedding through prostasin, *J Biol Chem.*
32. Tsai, C.-H. H., Teng, C.-H. H., Tu, Y.-T. T., Cheng, T.-S. S., Wu, S.-R. R., Ko, C.-J. J., Shyu, H.-Y. Y., Lan, S.-W. W., Huang, H.-P. P., Tzeng, S.-F. F., Johnson, M. D., Lin, C.-Y. Y.,

Hsiao, P.-W. W., and Lee, M.-S. S. (2013) HAI-2 suppresses the invasive growth and metastasis of prostate cancer through regulation of matriptase. *Oncogene*.

33. Kato M., Hashimoto T., Shimomura T., Kataoka H., Ohi H., Kitamura N., (2011) Hepatocyte growth factor activator inhibitor type 1 inhibits protease activity and proteolytic activation of human airway trypsin-like protease. *J Biol Chem151*, 179-187.
34. Cureton, D., Massol, R., Saffarian, S., Kirchhausen, T., and Whelan, S. (2009) Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization.*PLoS pathogens 5*, e1000394.
35. Klenk H.-D., Rott R., Orlich M., Bl√∂dorn J. (1975) Activation of influenza A viruses by trypsin treatment. *Virology 68*, 426-439.